

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s) : Koichiro YAMADA, et al. Examiner : Dr. Thomas C. McKenzie
Serial No. : 10/ 647,234 Group : Art Unit 1624
Filed : August 26, 2003
For : PYRIDOPYRIMIDINE OR NAPHTHYRIDINE DERIVATIVE

Hon. Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22318-1450

Sir:

DECLARATION

Dr. Kohei Kikkawa residing at 22-4, Kitaharadai 2-chome, Kawaguchi-shi, Saitama, Japan, sincerely and solely declare as follows:

(1) I am one of the co-inventors of the subject matter of the above-identified application and have complete knowledge of all aspects of the invention embodied therein.

(2) I graduated from Tokyo University of Science, Faculty of Pharmaceutical Science, Department of Pharmaceutical Science, Japan in March, 1981, and awarded the Master degree from Chiba university, Faculty of Pharmaceutical Science, Japan, in March 1983. And I was also awarded the Ph.D. from Chiba university, the same Faculty in March 1994.

(3) Since April 1983, I have been an employee of Tanabe Seiyaku Co., Ltd., residing 2-10, Doshomachi 3-chome, Chuo-ku, Osaka, Japan, and at the present time I am a researcher of the Pharmacology Research Laboratories in this company.

(4) Currently, I am a member of the following societies:

- American Heart Association (from 1998)
- The Japanese Circulation Society (from 1999)
- The Japanese Pharmacological Society (from 1981)
- The Pharmaceutical Society of Japan (from 1982)
- The Japanese Vascular Biology of Medicine (from 2004)

(5) Under my direction, the following experiments have been done.

Purpose :

This report investigates the relaxation of corpus cavernosum excised from rabbits of the test compounds disclosed in the specification.

Experiment : Relaxation of corpus cavernosum excised from rabbits

[Preparation of specimen]

New Zealand White rabbits (10-20 week old) were used. Immediately after the rabbits were killed by bleeding under anesthesia, corpus cavernosum was excised from the penis of the rabbits. After removing the connective tissue, the excised corpus cavernosum was cut into strips (length, about 5 mm) to give specimens. The strip specimens were mounted under about 1.5g. resting tension in Magnus tube containing a nutrient fluid* (10ml) and the tension to the specimens was stabilized over a period of about 60 minutes. After confirming the contractile response of the specimens in an aqueous solution of KCl having a high concentration (120mM), the specimens were subjected to the test. During the test, the nutrient fluid was maintained at $37 \pm 0.5^{\circ}\text{C}$ under gassing with 95% O_2 -5% CO_2 . The isometric tension was measured via U gauge and distortion amplifier (AP600 series, manufactured by Nippon Koden) and recorded on a pen recorder (Graphtec Multicorder MC6621).

*) Nutrient fluid contents: NaCl, 118.0 mM; KCl, 4.7 mM; CaCl_2 , 1.5 mM; KH_2PO_4 , 1.2mM; MgSO_4 , 1.2 mM; NaHCO_3 , 25.0 mM; glucose, 11.0mM; and EDTA, 0.023 mM.

[Relaxation in phenylephrine contractile specimen]

The specimens were contracted with phenylephrine. After the contraction of specimens became a continuous phase, the test compound was cumulatively added in an amount of 0.1, 1, 10, 100 and 1000 nM at an interval of 30 minutes. Finally, papaverine ($100 \mu\text{M}$) was added thereto, by which the maximum relaxation response was determined. The rate of relaxation of the test compounds in each concentration was calculated based on the maximum relaxation by papaverine ($100 \mu\text{M}$) which was counted as 100%. The relaxation rate of the test compound was evaluated by EC_{30} (i.e. an amount (nM) of the test

compound showing 30% relaxation rate to that of papaverine).

The results are shown in the following Table 2.

Table 2

Test Compounds (Example Nos.)	EC ₃₀ (nM)
1 [#]	2
7	1
14	9
32	6
39	5

#: the test compound was cumulatively added in an amount of 1, 10, 100 and 1000 nM at an interval of 30 minutes.

(6) The undersigned petitioner declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: April 27, 2005



Kohei Kikkawa



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s) : Koichiro YAMADA, et al. Examiner : Dr. Thomas C. McKenzie
Serial No. : 10/ 647,234 Group : Art Unit 1624
Filed : August 26, 2003
For : PYRIDOPYRIMIDINE OR NAPHTHYRIDINE DERIVATIVE

Hon. Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22318-1450

Sir:

DECLARATION

Dr. Kenji Omori residing at 6-8, Ryoke 2-chome, Urawa-ku, Saitama-shi, Saitama, Japan,
sincerely and solely declare as follows:

- (1) I am one of the co-inventors of the subject matter of the above-identified application and have complete knowledge of all aspects of the invention embodied therein.
- (2) I graduated from Kyoto University, Faculty of Agriculture, Department of Food Science and Technology, Japan in March, 1979 and was awarded the Master degree from the same university in March 1981. And I was also awarded the Ph.D. from the same university in May 1993.
- (3) Since April 1981, I have been an employee of Tanabe Seiyaku Co., Ltd., residing 2-10, Doshomachi 3-chome, Chuo-ku, Osaka, Japan, and at the present time I am a researcher of the Discovery Research Laboratories in this company.
- (4) Currently, I am a member of the following societies:
 - Japan Society for Bioscience, Biotechnology, and Agrochemistry (from 1980)
 - The Molecular Biology Society of Japan (from 1992)
 - The Japanese Biochemical Society (from 1997)
 - Biochemical Society (UK) (from 2003)
 - American Society for Biochemistry Molecular Biology (USA) (from 2004)

(5) Under my direction, the following experiments have been done.

Purpose :

This report investigates the Phosphodiesterase V (PDE V) inhibitory activity of the test compounds disclosed in the specification.

Experiment: Phosphodiesterase V (PDE V) inhibitory activity

[Preparation of PDE V]

A homogenate of the lung extracted from a male mongrel dog was centrifuged, and the supernatant was fractionated by an anion exchange column chromatography. The fractions satisfying the following conditions 1 to 5 were mixed to give a specimen of a partially purified PDE V.

Condition 1: It can selectively hydrolyze cGMP.

Condition 2: The cGMP hydrolyzing activity is not affected by EGTA*¹ or Calmodulin.

Condition 3: It's activity is not inhibited by a selective phosphodiesterase III inhibitor: CI930*².

Condition 4: It's activity is not inhibited by a selective phosphodiesterase IV inhibitor: Rolipram*³.

Condition 5: It's activity is inhibited by a selective phosphodiesterase V inhibitor: E-4021*⁴.

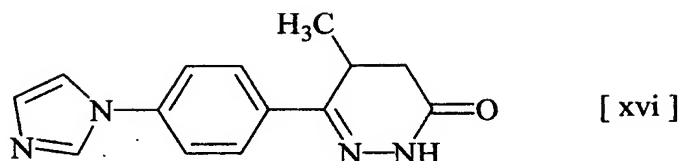
[Notes]:

*1 : EGTA is a calcium chelating agent having the following formula [xv]:



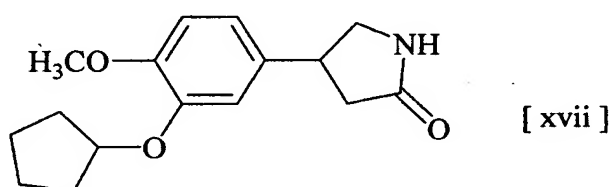
which can inhibit the activity of PDE I by chelating calcium ion in the enzyme solution.

*2 : CI930 is a selective PDE III inhibitor having the following formula [xvi]:



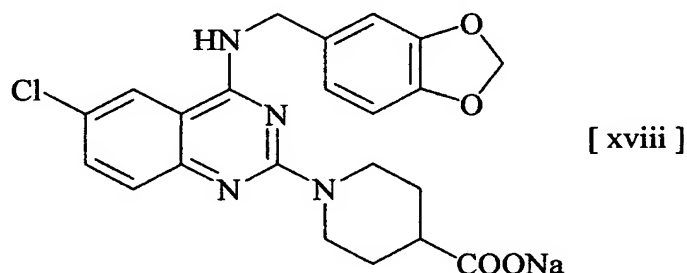
This is used for the purpose of confirming non contamination of PDE III in the PDE V enzyme solution.

*3 : Rolipram is a selective PDE IV inhibitor having the following formula [xvii]:



This is used for the purpose of confirming non contamination of PDE IV in the PDE V enzyme solution.

*4 : E-4021 is a selective PDE V inhibitor having the following formula [xviii]:



[Measurement of PDE V activity]

This was carried out by a partially modified method of the procedure of Thompson et al. (cf. *Advances in Cyclic Nucleotide Research*, Vol. 10, Raven Press, New York, pp. 69-92, 1979) as follows.

A specimen of partially purified PDE V was diluted with 50 mM Tris-HCl (pH 8.0) so that about 10 % of the whole substrate would be hydrolyzed. Said specimen (100 μ l) was added into a glass-made test tube. To the test tube was added an assay buffer (50 mM Tris-HCl, pH 8.0, containing 12.5 mM $MgCl_2$, 10 mM 2-mercaptoethanol) (200 μ l) and thereto further added a solution (5 μ l) of a test compound (compounds of Examples)(100 folds concentration) in dimethylsulfoxide. After incubating the solution at 37°C for 5 minutes, 200 μ l of

2.5 μ M [3 H] cGMP (307 kBq/200 μ l) were added thereto to initiate the reaction (final concentration, 50 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 4 mM 2-mercaptoethanol). After reacting at 37°C for 30 minutes, the test tube was moved into a boiling water bath to stop the reaction. 90 seconds later, the test tube was moved into an ice-water bath to cool the temperature of the reaction mixture to room temperature. After preincubating at 37°C for 5 minutes, an aqueous solution (100 μ l) of snake venom (1 mg/ml) was added to the test tube, and the mixture was reacted at 37°C for 30 minutes. The reaction was stopped by adding methanol (500 μ l) to the test tube, and the reaction mixture (1 ml) was charged into a column packed with Dowex resin (trade name: Dowex 1x8, manufactured by Sigma Inc.) (200 μ l). The resin was washed with methanol (1ml). The reaction solution passed through the column was combined with the washing solution, and the PDE V activity of the combined solution was determined by measuring the radioactivity of [3 H] guanosine in the solution.

The same procedure was carried out for a blank solution (only the assay buffer was used without adding the enzyme specimen) and also for a control solution which contained only the solvent (dimethylsulfoxide) instead of the test solution while an enzyme specimen was added.

The inhibitory rate of the test compounds was determined by calculating the rate of the inhibitory activity of each test compound to the inhibitory activity of the control solution, and the PDE V inhibitory activity was evaluated by IC₅₀ (i.e. an amount (nM) of the test compound showing 50% inhibitory rate to that of the control). The IC₅₀ was calculated by liner regression based on the inhibitory concentrations at 3 or more kinds of concentration of the test compound. The results are shown in the following Table 1.

Table 1

Test Compounds (Example Nos.)	IC ₅₀ (nM)
1	3.26
4	4.38
5	2.66
6	1.83
7	0.86
8	1.32
9	1.66

11	7.20
12	1.68
13	9.85
14	0.98
22	2.74
23	<1.00
29	<1.00
32	1.07
33	9.67
34	1.85
35	1.37
39	1.81
40	0.38
41	1.67

(6) The undersigned petitioner declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: April 21, 2005

Kenji Omori

Kenji Omori